21

N90-13943

POLARITY ESTABLISHMENT, MORPHOGENESIS AND CULTURED PLANT CELLS IN SPACE

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ABSTRACT

Plant development entails an orderly progression of cellular events both in terms of time and geometry (dimensional space). There is only circumstantial evidence that, in the controlled environment of the higher plant embryo sac, gravity may play a role in embryo development. We still do not know whether or not normal embryo development and differentiation in higher plants can be expected to take place reliably and efficiently in the micro g Space environment. It seems essential that more attention be given to studying aspects of reproductive biology in order to be confident that plants will survive "seed to seed to seed" in a Space environment. Until the time arrives when successive generations of plants can be grown, the best we can do is utilize the most appropriate systems and begin, "piece meal," to accumulate information on important aspects of plant reproduction. Cultured plant cells can play an important role in these activities since they can be grown so as to be morphogenetically competent, and thus can simulate those embryogenic events more usually identified with fertilized eggs in the embryo sac of the ovule in the ovary. Also, they can be manipulated with relative ease. The extreme plasticity of such demonstrably totipotent cell systems provides a means to test environmental effects such as micro g on a potentially "free-running" entity. The successful manipulation and management of plant cells and propagules in Space also has significance for exploitation of biotechnologies in Space since such systems, perforce, are an important vehicle whereby many genetic engineering manipulations are achieved.

Introduction. Since all biological development has evolved in the presence of an Earth 1 g vector, it may be argued that gravity plays a role in plant development. Edmund Sinnott even queried as far back as 1960 in his book "Plant Morphogenesis" whether the plant body as we know it could develop in the absence of specific gravitational stimuli or cues (Sinnott, 1960, p. 355). The term gravimorphogenesis is increasingly being used to designate the emerging discipline of the relationship of gravity to development. Some key questions as they apply to plants that need to be addressed include: "Do the cells of plants require gravity and/or other orienting forces at any stage in morphogenesis? What constitutes the or a minimal gravimorphogenetically responsive unit? Can totipotent cells function as a gravireceptor? Can pulses at certain g levels be enough to compromise or ruin a gravimorphogenesis-type experiment in Space or under microgravity conditions? etc.

By using test systems at different levels of initial organization, but which are capable of attaining or achieving the most advanced levels of higher morphogenesis, we should be able to evaluate and even pinpoint the threshold levels where the first detectable responses emerge. The Space environment offers unique opportunities to try to erase and to reapply g signals in proving the relationship of gravity to development. As opportunities for flight

experimentation increase, and especially as Space Station "Freedom" and other long duration near-0 g environments become available for gravimorphogenetic testing, the prediction is made that it will be proven that gravity is indeed a morphogenetic determinant.

Gravity and Embryo Development in Plants. The early cell divisions that partition the plant zygote into a multicellular tissue mass and lead ultimately to the orderly differentiation of organs are extremely important to organized development. Anatomical and morphological studies of embryogenesis in a variety of plants, both lower and higher, have demonstrated that the earliest division planes establish directionality for growth of the plant axis. The initial divisions are especially significant since their appearance often provides the first external sign that polarity has been determined. In certain plants polarity may be evident in the zygotic cytoplasm prior to the initial division, but for most plant embryos the axis of growth is fixed at the time the zygote is partitioned (cf. Wardlaw, 1955 p. 160; 1965a and b; Raghavan, 1986).

Much attention has been directed towards analyzing the phenomenon of embryo polarity but we still have little knowledge of the factors influencing the planes of early cell divisions. Also, nothing is yet known about the genetic regulation of polarity in plant embryos, and the relationship between molecular, cellular and environmental factors in establishing polarity is obscure. However, the bulk of available data support the thesis that initiation of polarity and determination of the plant axis is one of the earliest events in embryogenesis. The data further support the concept that factors influencing polarity can alter the development (cf. Wardlaw, 1955; Barlow and Carr, 1984).

Internal and external factors both play a role in determining polarity. For free swimming plant zygotes such as those of Fucus there is abundant experimental evidence that polarity can be influenced by a variety of environmental factors including light, temperature, nutrients, pH and mineral gradients (cf. Brownlee and Wood, 1986 and references there cited). There is, in addition, evidence that induced internal gradients can determine polarity. The development of zygotes in archegonia or embryo sacs is somewhat complicated by surrounding maternal tissues which is thought to influence polarity. (See also Willemse, 1981 for a discussion of polarity and megasporogenesis and megagametogenesis.) Whether or not the influence of the surrounding tissue is physical or physiological or both is not known. There is also evidence suggesting that treatments which affect the relationship between enclosed zygotes and surrounding tissue can alter polarity and subsequent development of the embryo.

Gravitational forces often have been observed to have profound influences on embryos of lower vascular plants (cf. LaMotte, 1937). Although many attempts have been made to assess accurately the role of gravity in the induction of embryo polarity and axis determination, the studies are generally inconclusive. In most of the work where centrifugation was used, stratification of the cytoplasm was commonly seen. However, in some cases the initial partitioning of the embryo and its later organization was altered, while in other cases there were no changes. Satisfactory control experiments were not always conducted and the significance of much of the published observations is not clear. In other studies zygotes were grown in various positions with respect to gravity or they were fixed in a substrate and grown on horizontal clinostats to determine if embyro orientation (development) was influenced. These studies are not sophisticated either in their design or in their execution but results often demonstrated that embryo polarity and the orderly segmentation pattern leading to normal development of the plant axis

were altered. Admittedly, there is insufficient evidence to permit any firm conclusions to be made concerning gravity effects on plant embryogenesis. Nevertheless, the preliminary data suggest that gravity may be important to normal embryogenesis and that plant embryo polarity, axis determination and pattern development could be adversely affected in Space.

Systems for Studying Embryogenesis in Space. A study of the influence of a microgravity environment on the early events of reproductive cell and zygote development would contribute substantially to a general understanding of regulatory factors in early plant morphogenesis. Equally important, results from such a study could provide a beginning for a clearer understanding of the behavior of plants grown in the environment of Space (cf. Keefe and Krikorian, 1983; Krikorian et al., 1984; Halstead and Dutcher, 1987). For this type of developmental analysis, intact flowering plants would in my view be the preferred material to study but this is not readily feasible because of the current lack of reliable information concerning most aspects of their reproductive biology in the Space environment. For most flowering plants nothing is known about pollen tube growth, sperm cell migration and the fertilization mechanism as they occur in a microgravity environment (cf. Halstead and Dutcher, 1984, 1987 and references there cited).

Also, and for the foreseeable near-term, duration of Space flights will be relatively short and thus the possibility of carrying out a "seed to seed to seed" type of experiment (cf. Keefe and Krikorian, 1983; Krikorian et al., 1984) will not be possible even using a so-called tachyplant or fast-cycling plant such as the Crucifer Arabidopsis (cf. Ivanov, 1974).

Our approach, therefore, has been to use cultured plant cell systems which are capable of undergoing organized development (i.e., somatic embryogenesis) in vitro. Such systems provide several advantages. These include the fact that large numbers of cells and organizing units can be manipulated for experimentation. Excision of developing plant embryos from seeds in equivalent numbers would be very difficult, if not impossible. Certainly, removal of fertilized eggs or zygotes from the embryo sac in the ovule of higher plants is out of the question. Indeed, it will be a landmark achievement when a zygote so removed can be nurtured to full maturity. In addition to such practical considerations, we have adopted the view that in vitro systems involving totipotent or morphogenetically competent cells present other advantages for proving questions involving higher plant development--especially in Space. Free cells in vitro, unlike cells in the strictly controlled environment of the embryo sac in ovules should be more responsive to perturbations such as those that might exist in micro g. We hypothesize that there should be no highly controlled environment other than that extant in the "genetic program" (whatever that may really mean) of the test system. Here, unless the developing cells and proembryos are maintained in vitro in an environment of strict balance of nutritional and other factors, there is a chance (as in the case in over-enrichment) of massive proliferation of undifferentiated tissue being formed, or in the case of impoverishment, a great chance that proper growth or differentiation might not occur. Between the extremes lies the "optimum" set of gradients for the differentiation of tissues and organs to occur. In short, we feel the exaggerated potential for expression of plasticity of development and growth in in vitro systems, such as those involving totipotent free cells, should provide a valuable means to probe environmental and nutritional impacts as developmental expression responds to, and reflects, complex interactions such

as may be encountered in Space, and where precise developmental signals may be altered (cf. Jennings and Trewavas, 1986; Schlictling, 1986).

Cosmos Carrot Cell Culture Results Work done at Stony Brook in connection with Cosmos 782 and 1129 using totipotent carrot cells which could undergo somatic embryo formation showed that while the broad events of non-sexual embryogenesis could and did occur, problems remained. In the first instance, the carrot cell system we used for the Cosmos experiments involved the generation of so-called competent units, their induction on Earth so as to produce what are termed in botanical embryological parlance proembryos, and their subsequent exposure to Space conditions so as to evaluate their capacity to express further developmental capacity. The fine point of detail to be appreciated is that the cells used were already developmentally determined, and, by prior experience, shown to be capable of undergoing somatic embryogenesis. They were not manipulated to achieve their morphogenetic capability in Space. Since programmed cells, as it were, were generated on Earth, and chilled to preclude further development into embryos on Earth, we have argued that they could well have retained a "memory" of the Earth's g environment. How one might successfully "erase" such a "memory" is a moot point but it can be proposed that for a start, successive generations of morphogenetically undetermined plant cells should be grown and induced in Space in a micro g environment. The second criticism to be raised is that the Cosmos 782 experiment was not repeated on the Cosmos 1129 flight. A third is that none of the materials was fixed in flight. Only after satellite recovery and transport of samples to Moscow was fixation performed. Even now, only preliminary presentation has been made because of reluctance to publish inadequately repeated experiments (cf. Krikorian and Steward, 1978, 1979; Krikorian et al., 1981). For the purposes of making a point and in the context of this presentation, reference may be made to calculations carried out on data derived from 1 g centrifuge and micro g controls (cf. Tables 1 and 2). Here, the results of scoring the normalcy of the developmental pathway of competent cells and proembryonic units to later stages of embryogeny is presented. The transition from one embryonic stage to another was slowed down. Specifically, in micro g, a greater proportion of embryos were at "stage 2" and fewer embryos had progressed to "stages 3" or "4."

Theimer et al. (1986) using a system somewhat similar to carrot (they used anise, Pimpinella anisum) have reported increased biomass of embryonic structures generated in Space in liquid cultures. Most of the criticisms of experimental protocol raised above for our carrot experiments apply to their work with anise as well, however, and for me, their results remain arguable and equivocal as well. Surely much more work will be needed to resolve unanswered questions.

A much improved assay system for carrot is in the process of being developed at Stony Brook and will provide a much better opportunity to get definitive answers to questions as to whether development of cultured plant cells in Space can occur with acceptable fidelity from a morphological, cytogenetic and temporal perspective (cf. Smith and Krikorian, 1988). Not only will answers gotten from such systems be of interest to developmental plant biologists but they will have significance for those seeking to use biotechnological procedures and manipulations in Space for a variety of reasons (cf. Keefe and Krikorian, 1983). Indeed, the ability to use and manipulate cells and other kinds of propagules in vitro reliably in Space will be a necessary prerequisite to many projected or hypothesized commercialization schemes (cf. Krikorian, 1985).

Commentary. The foregoing seeks to emphasize therefore that there is much that we do not know about plant cells and how they behave in Space. Finally, and with no intention of detracting from the importance of studies seeking to obtain answers to such important questions as: To what extent does the gravitational environment influence polarity, axis determination and embryogenesis in vascular plants? Are the haphazard positions of the embryos and the abnormalities noted in megaspores grown on clinostats actually related to the effect(s) of g neutralization? Is the biochemical relationship between the embryo and nutrient supply - whether in situ, in maternal tissue or in vitro in appropriately designed culture vessels or apparatuses designed to provide "all" the "right" signals - influenced by the Space environment? Also, the less sophisticated but perhaps more compelling questions arises as to whether we have satisfactory and convincing answers as to whether we yet have the means available to grow intact plants over protracted periods in Space. We have made some interesting observations on decreased levels of cell division in roots after they have grown for a week in Space, we have also observed chromosome aberrations such as fractures and breaks in cells of roots grown in Space for relatively short periods. There is much to suggest that we have a long way to go before we can be confident of being able to grow plants through successive generations (cf. Krikorian and O'Connor, 1984; Halstead and Dutcher, 1987). We have no reason to suppose that results of extended duration experimentation will not disclose or exaggerate responses such as those alluded to and that are merely suggestive and inconclusive at this time.

Acknowledgements. The research described in this presentation was supported from grants from the National Aeronautics and Space Administration. A broadly based research effort entitled "Cells, Embryos and Morphogenesis in Space," NASA NSG 7270 has permitted a range of aseptic test systems to be developed with the view of eventually exposing competent systems at varying levels of prior organization to the Space environment.

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Table 1

Contingency Chi-square Method of Analysis for Somatic Embryogenesis in microgravity and on a 1 g centrifuge in Space. Stages of embryo development were subjectively categorized as Stages 1 to 4. Analysis from data of Krikorian and Steward (1978).

	-	0 g	1 g	Σ	% of Total
Stage 1 (Heart Shaped)	Obs.	6105	5655	11760	67.70
	Exp.	6103.16	5656.34	11759.5	
	Dev	+ 1.84	- 1.34		
	χ^2	0.0006	0.0003	0.0009	
Stage 2 (Torpedo shaped, <.75 and 1.5 mm long)	Obs.	1680	1345	3025	17.42
	Exp.	1570.41	1455.44	3025.85	
	Dev.	+109.59	- 110.44		
	χ^2	7.65	8.38	16.03	
Stage 3 (Advanced embryonic forms with distinct root between .75 and 1.5 mm long)	Obs.	760	865	1625	9.36
	Exp.	843.80	782.02	1625.83	
	Dev.	- 83.80	+82.97		
	χ^2	8.32	8.80	17.12	
Stage 4 (small plantlets with well developed root, > 1.5 mm)	Obs.	470	490	960	5.53
	Exp.	498.53	462.03	960.56	
	Dev.	- 28.53	+27.97		
	χ^2	1.63	1.69	3.32	
Σ	Obs.	9015	8355	17370	100.01
	Exp.	9015.90	8355.84	17371.74	
	Dev.	- 0.90	-0.84		
	χ^2	17.60	18.87	36.47	
	% of total	51.90	48.10		

Chi-square χ^2 = $'(Obs-Exp)^2$ = 36.47
Degrees of freedom = (2-1)/Exp (4-1) = 3
$p < .001$
Table χ^2 (df3, P.001) = 16.27

Method of calculating expected values
Exp (Stage 1, 0g) = (% of total for Stage 1) (Total in 0 g)
= (.6670) (9015) = 6103.16 etc.

Table 2

Comparison of degrees of embryonic development (stages 1 to 4) achieved by totipotent cells of carrot at 0 g and 1 g. Since the total number of plants at 0 g (9015) and 1 g (8355) were unequal, use was made of a contingency chi-square test. Analysis from data of Krikorian and Steward (1978).

	OBSERVED (PERCENT OF TOTAL)		
	0 g	1 g	Difference
Stage 1 (Heart shaped)	67.7	67.7	0
Stage 2 (Torpedo shaped < .75 mm long)	18.6*	16.1	+2.5
Stage 3 (advanced embryonic forms with distinct root between .75 and 1.5 mm long)	8.4	10.4	-2.0
Stage 4 (small plantlets, with well developed root, > 1.5 mm)	5.2	5.9	-0.7
Σ	99.9	100.1	

*At 0 g, a greater proportion of plants were still at stage 2, and fewer plants had progressed to stages 3 or 4. $P < .001$!